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Cultured Neuron Probe

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This QPR is being sent to you before it has been reviewed by the staff of the Neural Prosthesis Program

General Introduction

Our aim is to develop a cultured neuron probe. This consists of a silicon structure into which individual dissociated neurons can be placed, and which can be inserted into an intact nervous system. Furthermore, within the structure each neuron is in close proximity to an electrode, by means of which it can be stimulated, or its activity can be recorded, through electrical leads which connect to external electronics. It is hoped that neurons in the probe will synaptically integrate with the host nervous system, to provide a highly specific, relatively non-invasive, two-way communication channel. If this occurs, the methodology has important possibilities for neural prostheses. The goal of this project is to perform initial experiments to establish the feasibility of communication by means of a neuron probe. The tissue we have chosen in which to initially implant the probe is the rat hippocampus. If initial studies are successful, probes will be designed and implanted for communication with sensorimotor cortex.

The neuron probe we plan to fabricate will be made of micromachined silicon and will have sixteen electrodes: one conventional electrode to detect activity during placement of the probe, and fifteen within wells into which neurons will be placed. Its configuration will be similar to that of passive multielectrode probes which have been developed. It will be implanted when the cultured neurons are very young, and after a time of weeks it is hoped that they will have survived and made two-way synaptic connections. By stimulation and recording in the host and probe, we will test for the existence of such connections. An essential feature of the experiments is that the viability of the implanted neurons will be independently determined over time by stimulating them and recording their resulting action potentials.

Summary

During the last quarter, progress was made in all parts of the project, as summarized below:

In experiments with SCG neurons, the new well and grillwork design was shown to promote rapid and easy outgrowth of processes from cells in the wells. Survival for up to several weeks was observed, with good growth and differentiation of processes into substantial dendrites. The protocol for DiI staining was refined, and beautifully stained fine processes were seem soon after plating. After 6 days, the stain was still visible. Scanning electron microscope techniques were refined so that cell bodies and processes growing from them out of wells could be more clearly seen than with optical microscopy.

In order to facilitate tests of the recording capabilities of neurochips, techniques were developed for successfully penetrating cells in wells intracellularly with a glass micropipette. These cells could be stimulated intracellularly to fire action ptentials, and the recording electrode in the well simultaneously observed to see the extracellular signal. In the first attempt with a good neurochip, no signals were seen, which led to the discovery of a serious shunt capacitance problem. During the evolution of the neurochip design, the insulation under the bonding pads had changed from 1 μm silicon dioxide to 0.1 μm silicon nitride, and the shunt capacitance in the latter case was ten times the electrode coupling capacitance. This attenuated the signal by an order of magnitude. A procedure for correcting this problem on the present batch of neurochips, by eliminating the pads and connecting to the fine leads, is being developed. Future chips will have smaller pads, or thicker insulation, or both.

The culture system for hippocampal neurons was refined, so that survival for four weeks in culture is now routine. One problem was that Ph excursions when cultures were removed for viewing were causing cell death. Another was that over a long period the osmolarity of the medium was changing significantly, as the cultures are fed by only changing half the medium. After attention to these problems, the survival was much improved.

Hippocampal neurons were found to grow and survive well on neurochips with the new grillwork design. However, they invariable escaped through the corner hole out of which their axon emerged, after two days or less. It is not known whether this would occur with a probe *in vivo*, but to be safe, and to make *in vitro* experiments possible, new grillwork designs with smaller holes around their periphery are being developed. Dummy neurochips are being made with four different hole sizes and patterns, for tests to be performed in the coming quarter.

Hippocampal neurons labelled with DiI have been plated onto long-term slice cultures. It was found that observation of their growth into the slices caused death arising from phototoxicity of DiI. Various ways of ameliorating this problem are being explored. However, observation of these neurons with a new two-photon confocal microscope has been done successfully, and good axonal growth through the entire slice thickness was seen. This microscope may induce much smaller phototoxic effects, but detailed studies remain to be done.

Grafted hippopcampal neurons stained with DiI have been observed after 6 months *in vivo*. The stain was still visible on cell bodies and dendrites, with standard fluorescent microscopy. Background staining was a problem, which might be reduced with confocal microscopy. Implants of neurons in new dummy probes that have the good grillwork design will follow in the coming quarter.

Neurochips

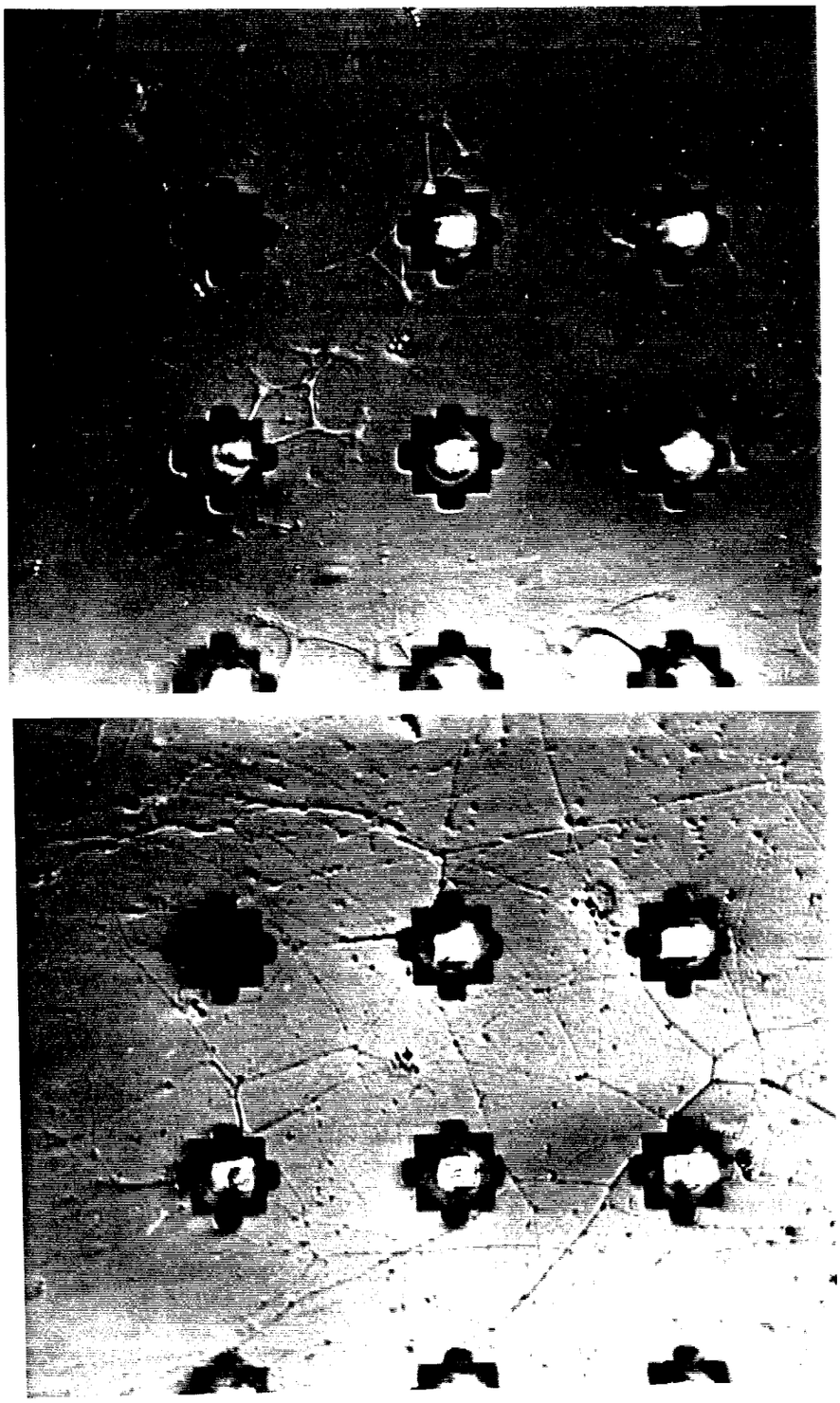
SCG neuron experiments

Outgrowth and long-term survival

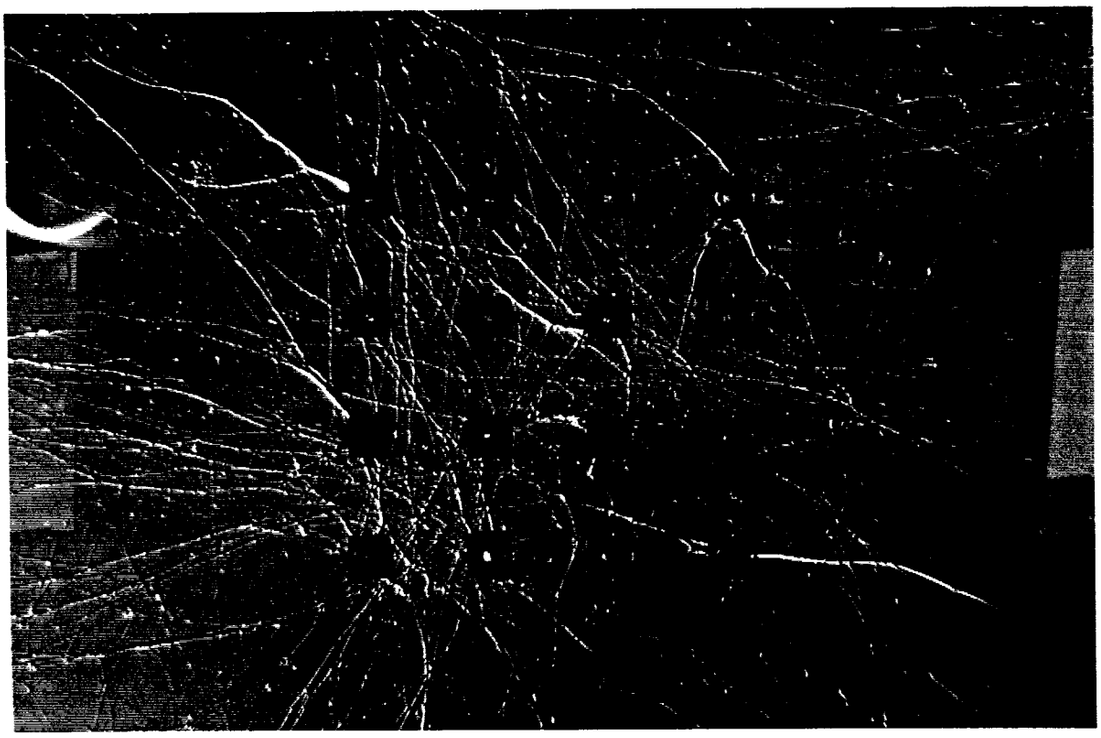
During this quarter we have continued to monitor the initial outgrowth and long-term survival rates of neurons in the wells of both dummy chips (without electrodes) and real neurochips (with electrodes). The new, overhang-free grillwork design described in the last report allows process outgrowth by a majority of cells; however, the current designs also allow neurons to "escape" by movement of the soma out of a corner (or occasionally the central) hole. Furthermore, some neurons placed into wells apparently float out again before attaching. Since hippocampal neurons (plated in Neurobasal medium), unlike SCG neurons (plated in L15-based medium), stick down very quickly upon settling, it may be possible to improve adhesion of SCG neurons by plating in Neurobasal rather than L15 medium.

Four dummy chips were loaded with neurons. In three successful cases, four, seven, and thirteen neurons sent out processes within a day of being loaded into wells. (In the other case, all neurons in the dish died for unexplained reasons.) After a day in culture, all other wells on these chips either were empty or had neurons crawling out of the corners. On the chips with seven and thirteen neurons growing out initially, five and six wells respectively had thick dendrites emerging at two weeks post-plating. The Nomarski photographs on the following page show good outgrowth after 6 hours, and fast process growth in tyhe first three days.

Outgrowth and survival were just as good on real neurochips from the latest production run. Three such chips had outgrowth from seven, five, and eight wells one day after loading; after two weeks, there were dendrites emerging from three, four, and four wells respectively. The Nomarski photo below, taken after two weeks in culture shows good long-term survival, with dendrites from 6 wells and thick processes from two others.



SCG process outgrowth from several neurons in wells of a dummy chip, six hours (top) and three days (bottom) after loading.



The movement of growing neurons out of wells is still problematic. Some neurons crawl through corner holes a few days after loading; this problem should be alleviated by the new grillwork designs currently in production. Other neurons appear to ooze out of wells even after a couple of weeks in culture; they appear to grow too large to be contained within the wells. This should not be a problem for the smaller hippocampal neurons.

Staining neurons in suspension

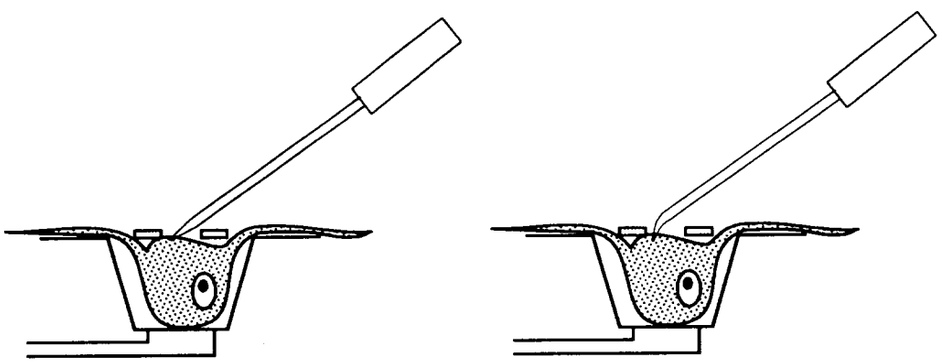
Visualization of neuronal outgrowth from wells is facilitated by staining with a membrane-bound dye such as DiI. We now have a reliable protocol for staining dissociated SCG neurons in suspension with C12 DiI before plating. This protocol consists of staining for 20 minutes in 40 μg/mL DiI, followed by rinsing the cells by spinning them twice at low speeds with a "cushion" of bovine serum albumin at the bottom of the tube.

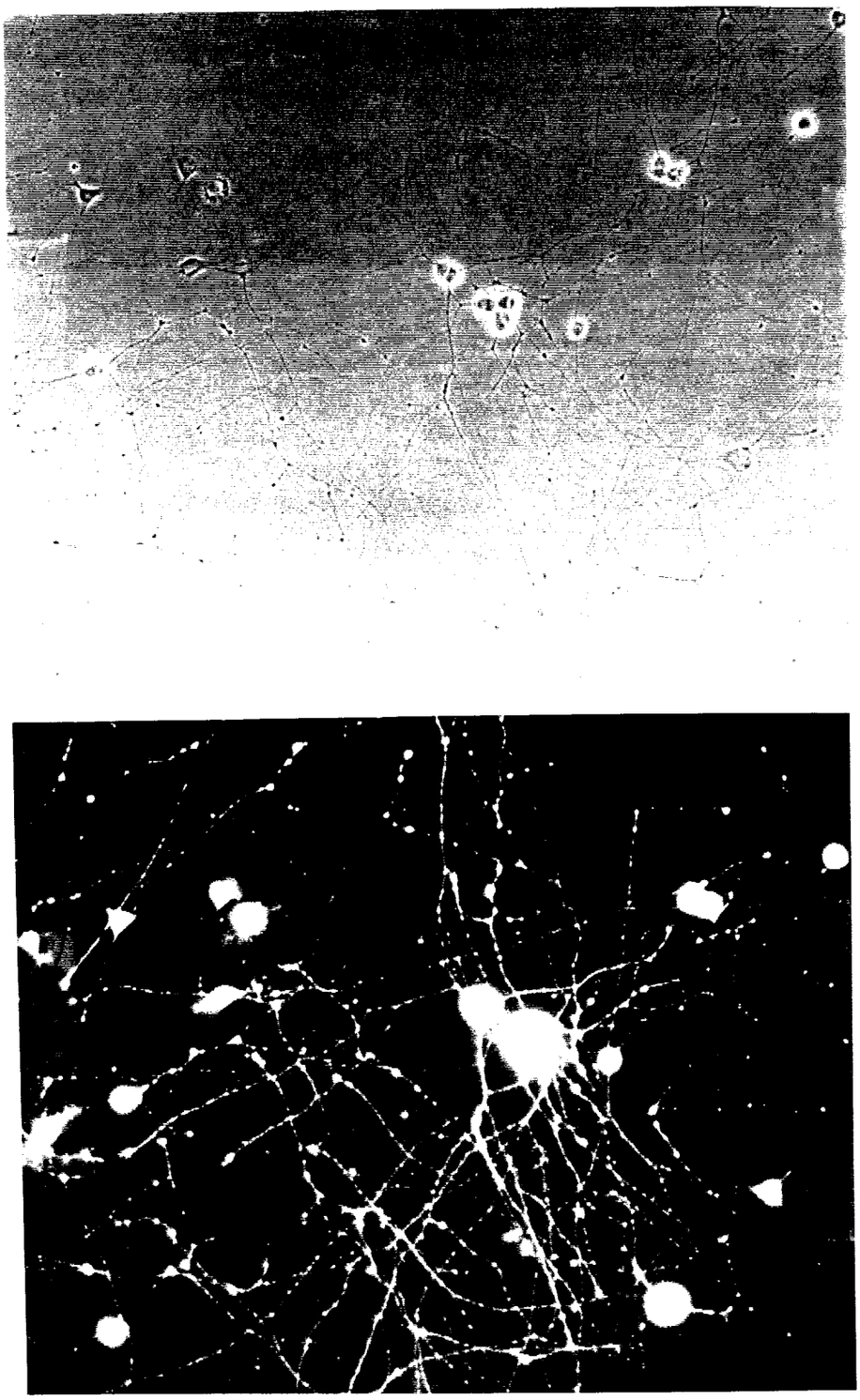
This protocol results in bright staining of all cells in the culture. Even the finest structures of growth cones are visible a few hours after plating. After about three days, the dye starts to be internalized into vesicles, resulting in diffuse staining of the cell body and any thick processes, and faint staining of the fine processes. Phototoxicity of DiI in SCG cultures remains to be tested.

The photos on the two following pages illustrate the staining in day-old and six-day-old cultures.

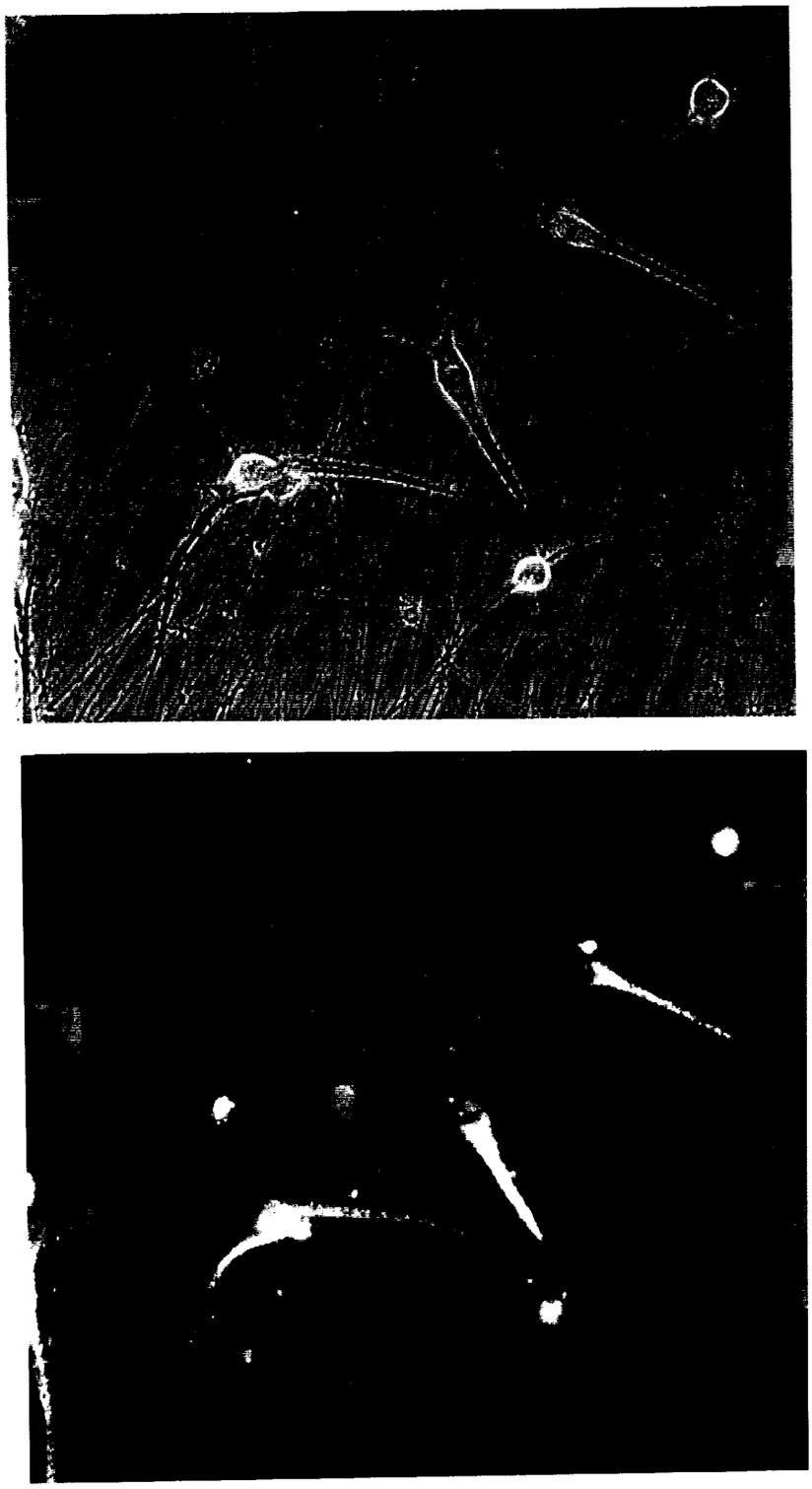
Electrophysiology on cells in wells

In order to demonstrate that the neurochip electrodes can reliably record and stimulate action potentials in neurons in wells, it is necessary to measure independently the electrical activity of the neurons. The most straightforward way to do this is to record from cells in wells simultaneously with the neurochip (recordings acquired by the LabVIEW instrument, MultiElectrode, on the MacII) and with an intracellular electrode (recordings acquired on a storage oscilloscope). Because the neurochip is opaque, it is necessary to do the intracellular recording on an upright microscope, and the position of the objective places a severe constraint on the angle of approach of the intracellular electrode (see diagram below). However, it was verified with neurons in control dishes that intracellular penetrations could be made successfully even with the intracellular electrode at a shallow angle (about 30°) relative to the substrate, as shown at left in the figure.





Phase (top) and fluorescence (bottom) micrographs of the same SCG neurons in a day-old culture. Neurons stained in suspension with C12 DiI before plating. Fine processes that are barely visible under phase are readily visible under fluorescence.



Phase (top) and fluorescence (bottom) micrographs of the same SCG neurons in a six day old culture. Neurons stained in suspension with C12 DiI before plating. Most of the dye-labeled membrane has been internalized and is visible only in the somata and dendrites.

A first attempt to record intracellularly from cells in wells was performed on a 17 day old culture on a dummy chip. Successful penetrations were made into all five neurons that were still in wells (as determined by the presence of dendrites). All had fairly low resting potentials, ranging from -20 to -40 mV. Penetrations were maintained for three to seventeen minutes. All neurons fired small (about 20 mV) anode break action potentials after a hyperpolarizing current pulse of a few nanoamperes, and one cell fired action potentials when depolarized.

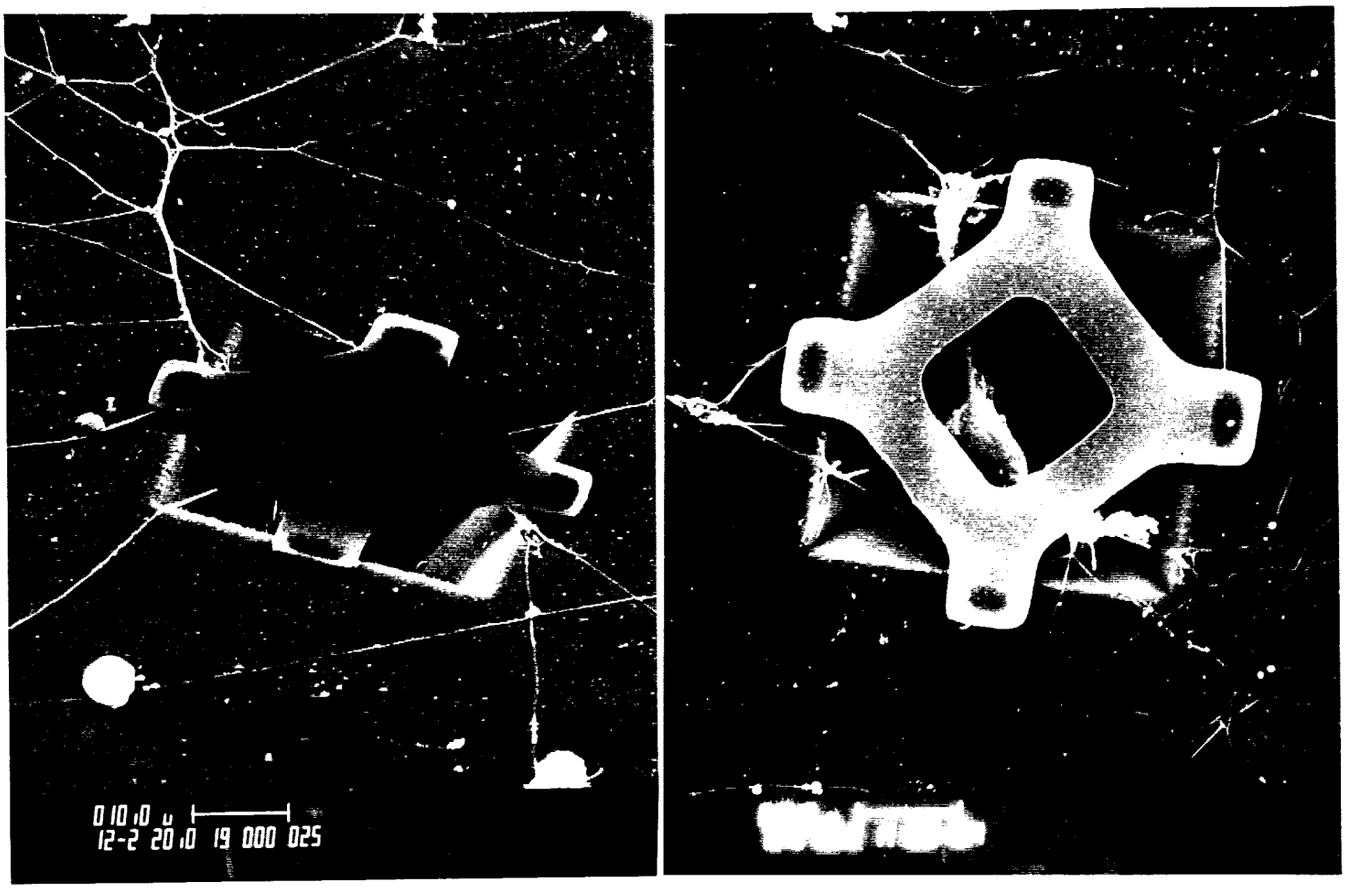
The second intracellular recording attempt was made during the testing of a real chip (see next section). In this 12 day old culture, dendrites appeared to be emerging from five wells, though on closer examination it was discovered that in two cases the neurons were sitting on top of the wells. Successful penetrations were made into all five neurons and anode break action potentials were evoked from them all.

When intracellular recordings were made from cells in wells, the resting potentials were lower (less negative) and the cells less likely to spike reliably than during recordings made from similar cultures on an inverted microscope. This may be due to the shallow angle made by the electrode with the substrate, which might cause a more ragged hole to be torn in the cell membrane. This problem might be alleviated by the use of intracellular electrodes bent at the tip by placement near a heated platinum filament. The bulk of such an electrode would be at the same angle as before, but the tip of the electrode would approach the cell at a much steeper angle (see diagram at right above).

Scanning Electron microscopy

In order to closely observe neurons growing from wells, it is desirable to image them with scanning electron microscopy. To do this, cells are fixed in 2% glutaraldehyde in PBS at 4° C. for one hour, fikkowed by postfixation in 1% OsO_4 in PBS on ice for 1 hour. The sample is then dehydrated in an ethanol series and then critical point dried and coated with gold. The pictures below show views of two neurons fixed one day after plating. Fast outgrowth of

multiple axons is seen. The cell body is concealed by the grillwork at left, but visible in the right-hand photo. The dehydration causes shrinkage of cell bodies to perhaps half their size.



Electrophysiology on full chip Q1

We recently received a set of 17 complete chips, designated the 'Q' series, from the Tai lab. These chips have electrodes, silicon nitride grillwork, and no overhang. We mounted four of these chips and grew SCG neurons in their wells. After 2 weeks in culture, chip Q1 had 5 live cells remaining in wells. We first tested a sister control culture, to determine our success rate at obtaining intracellular penetrations. Of ten cells tested, we were able to get stable recordings on nine. These all had -40 mV resting potentials and spiked on anode break to +10 mV. Four of these cells would also fire upon depolarization.

We then hooked up chip Q1. We were able to get stable penetrations into all five cells in the wells, and we were able to fire action potentials in all cells. However, we were unable to detect this activity from the chip electronics. This failure was traced to a design flaw in the chips, which will be discussed below.

Theoretical signal sizes:Response to current sources

Since the well surface is backed by silicon dioxide, to first order no current flows through the well. Then the entire well volume is an isopotential, and the electrode will sense the average voltage across the top surface of the electrode. This assumption will fail when the current source is extremely close to or inside of the well, where the resistance of the paths through the well become important. The resistance estimates found using this assumption will thus be larger than the actual values.

Using this assumption, the voltage due to a current source I at a distance r from the top center far from the well is $V(r) = I\rho/2\pi r = I(25\mu m/r) \times 4.5\,k\Omega$, where ρ=70 Ω-cm is the resistivity of the saline. For a current source at the top of the well center, $V = 2I\rho[\ln(1+\sqrt{2})]/\pi a = I \times 13.3k\Omega$, where *a*=30 μm is the size of the well. For a current source within the well a distance t from the top, an additional resistance $R = \rho/2\cos\theta[(a-2t\cos\theta)^{-1} - a^{-1}]$is encountered, where θ is 54º, the angle of the well sides. This amounts to 29 kΩ at the bottom of a 15 μm deep well. We confirmed these numbers using two 1 μm diameter pipettes, one as a current source and one as a sensor. Placing the two pipettes in various relative positions in the chip filled with saline, (including having the sensor at the bottom of a well), we found that the measured voltages come within a factor of two of these simple estimates.

So, the worst-case scenario for chip Q1 would be that a cell acts as a 10 nA current source at the top of the well. Then, the electrode at the bottom of the well should see a 130 μV signal, easily distinguishable from the noise level.This signal is increased to 430 μV if the current source is in the center of the well, and to 730 μV if it is at the bottom.

We tested the preamplifiers, amplifiers, and digitizers by injecting a voltage signal into the preamplifier. The calibration of the digitizer in this situation is 1.8 μV/count, roughly what is expected, considering all the gains in the system. By floating the entire saline bath with a sine wave potential injected through the Ag/AgCl electrode, we measured the same calibration of 1.8 μV/count.

Using a pipette electrode as a current source, we injected 1 μA of current 25 μm away from the top center of a well. The signal we measured through the chip electronics was only 400 μV, a factor of 10 too small.These results indicate that the signals do exist within the well, and are not attenuated or absorbed by the silicon. However, some element must exist which prevents the signals from reaching the preamplifier.

Impedance measurements

By measuring the impedances of the electrodes in various configurations, we were able to separate the different elements of the circuit. We found that in addition to the expected 70 pF of parasitic shunt capacitance due to the external wiring, we also had approximately 400 pF from each electrode to the silicon substrate (which has a very low impedance to ground when the chip is in saline). The signal V_1 measured at the preamp relative to the initial signal size V_0 when driven by an electrode capacitance C_e in the presence of a shunt capacitance C_S is $V_1 = V_0 C_e / [C_e + C_s]$. Since the bare gold electrodes normally only have about 50pF of capacitance coupling the electrode to the bath, this huge shunt capacitance attenuates the signal by a factor of approximately 10.

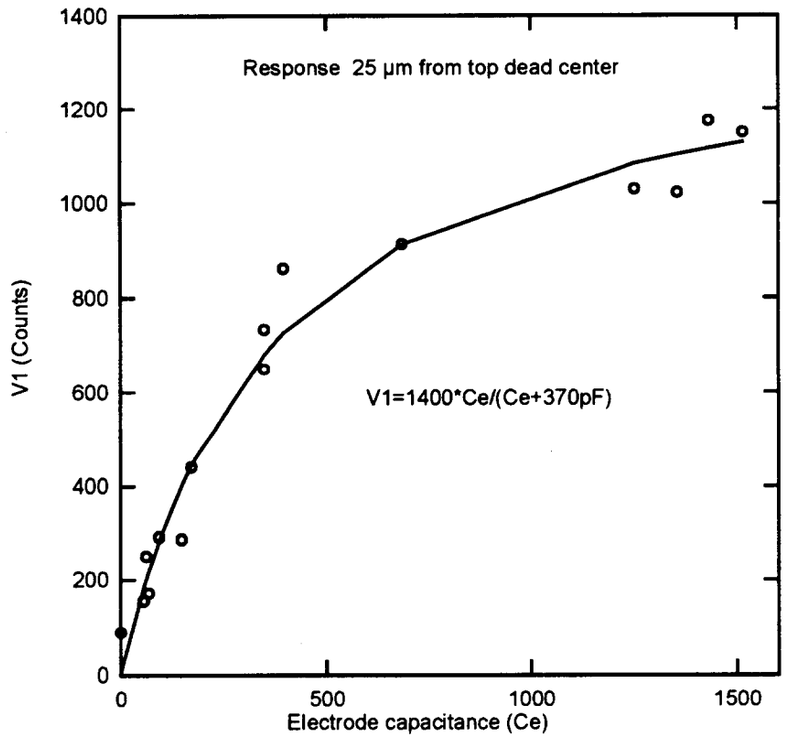
After several design changes, these chips have 1000 Å of silicon nitride separating the gold chip wiring from the silicon. The wires themselves are approximately 1 cm long by 10 μm wide, for 0.01 mm^2 of area. The bonding pads are 1.0 mm wide by 1.5 mm long, for 1.5 mm^2 of area. The capacitance $C = \varepsilon A / 2d$, with ε_0=8.85x10^{-12} F/m and ε_r=7 for Si_3N_4 is 460 pF for the bonding pads and 3 pF for the wiring. So, the conclusion is that the bonding

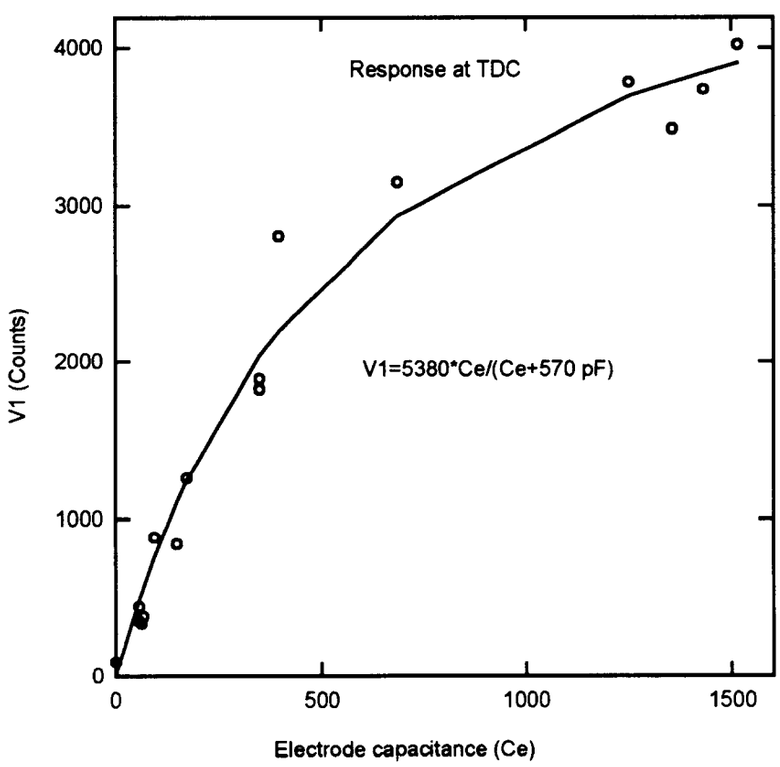
pads short the signal to the silicon. (In the original design the bonding pads were insulated from the silicon by approximately 1 μm of silicon dioxide, yielding a shunt capacitance more than ten times smaller.)

To test this theory, we varied C_e by platinizing the electrodes to varying degrees on chip Q4. We were able to increase the capacitance up to 1500 pF, 3 times as great as the shunt capacitance. The response of the well electrodes to a 1 μA, 500 Hz wave was measured using the MultiElectrode program. Current was injected at the top dead center (TDC) of the well, as well as at 25 μm away from TDC. The height of the pipette was accurately determined by gently touching the nitride grillwork, to ensure the bottom of the electrode was level with the top of the well. The small response of the wells when the pipette was next to the ground electrode was subtracted off. We used a least-squares analysis routine to fit the data (see the graphs on the following page) to the equation $V_1 = V_0 C_e / [C_e + C_s]$. For the pipette at TDC, V_1 =9.7 mV and C_S=570 pF yields the best fit. For the pipette 25 μm away, the best fit is given by V_1 =2.5 mV and C_S=370 pF. Using the first-order analysis above, we would expect V_1=13.3 mV at TDC and V_1 =4.5 mV at 25 μm. Thus, the signal is within a factor of 2 of the expected size, and platinizing the wells overcomes the effects of the shunt capacitance.

Table I. Comparison of measured and calculated pulse sizes as measured at a well electrode, with a 1 μA point current source at various positions relative to the well.

	Predicted.	Measured	
		by well electrode	by pipette
well bottom	42 mV		
TDC	13 mV	9.7 mV	
at 25 μm	4.5 mV	2.5 mV	5.1 mV


Response 25 µm from top dead center
V1=1400*Ce/(Ce+370pF)
V1 (Counts)
Electrode capacitance (Ce)
0
200
400
600
800
1000
1200
1400
0
500
1000
1500


Response at TDC
V1=5380*Ce/(Ce+570 pF)
V1 (Counts)
Electrode capacitance (Ce)
0
1000
2000
3000
4000
0
500
1000
1500

Fixing the chips

The most obvious solution to the shunt capacitance problem is to platinize the electrodes. Unfortunately, the increase in capacitance decays rapidly; after 7 days, the capacitance of the most heavily platinized wells had fallen by a factor of 10, to 150 pF. Such a low capacitance will cause the signals to be attenuated by a factor of ten. Since we want to use the chips in culture for many weeks, we must eliminate the large shunt capacitance. In the future, the chips will be designed to reduce the shunt capacitance to the silicon by reducing the pad area. In order to use the present batch of chips, however, improvised measures must be taken.

We have successfully eliminated the shunt capacitance problem in one chip thus far by etching off the bonding pads and directly attaching 40 μm diameter gold-plated tungsten wire to the 10 μm wide gold lines on the chip using indium solder. The indium solder contacts the nitride surface in 200-500 μm diameter circles, for areas of 0.03-0.2 mm^2 and shunt capacitances to the silicon of 10-60 pF. Measured shunt capacitances range from 10-40 pF to the silicon, and approximately 50 pF for the wiring and preamp, while electrode capacitances are approximately 30 pF before platinization and 250 pF when freshly platinized. This chip should give acceptable losses in signal size; unplatinized electrodes should see an attenuation approximately by a factor of three, while platinized electrodes may have an attenuation of less than a factor of 2.

This is an extremely difficult process, so the yield and production rate are extremely low. Full quantitative measurments have not yet been made on Q6; but we expect to test and grow cells in it in the next quarter, and to produce more low-shunt chips from our stock.

Hippocampal neuron experiments

Survival and growth of hippocampal neurons in chip wells with no overhang was tested. We found that cells pushed into wells grew normally with

over 50% survival, but that all cells invariably escaped through well corners within two days. Pictures of the cells escaping show that they easily squeeze through the corner holes, which are essentially 10 μm triangles. The neurons tend to escape by following the quickly-developing axon, which is known to exert a large tension on the cell body. Torre and Stewart [J. Neurosci., 12:762-772 (1992)] demonstrated that rat hippocampal neurons can easily migrate through 5 and 8 μm diameter nominal pore-size Nucleopore filters, but not through 1, 2, or 3 μm size filters. In the next quarter, we will be testing dummy neurochips with new grillwork designs that have smaller holes, to find a geometry which will better confine the neurons to the wells.

We have also made two significant discoveries which have allowed us to increase the length of time we are able to keep cultures alive and healthy from 4 days to 3 weeks. First, following Laufer and Gross [Soc. Neurosci. Abs. 20:1202 (1994)], we found that high pH even for short periods can cause significant cell damage. We routinely removed cultures from the 5% CO_2 incubator for extended periods of time, believing that high pH did not affect the cells. Laufer and Gross reported a threshold at about pH 7.7, above which the cells spontaneously fired action potentials until they died. We found that incubated Neurobasal+B27 in 35 mm tissue culture dishes reaches pH 7.7 in about 10 minutes after removal from 5% CO_2. We also found that high pH does not appear to affect the cells until about 4 days in culture, before which the cells are presumably not active or not sensitive to high pH. Thus, our routine now is to keep the cells in a 5% CO_2 atmosphere except for brief observation periods not to exceed 10 minutes, and to abstain from opening the incubator door more often than every ten minutes (twice the approximate recovery time for our incubator).

The second advance is to monitor and control the osmolarity of the tissue culture medium. Neurobasal+B27 normally has an osmolarity of 220 mOs/kg, significantly lower than the normal tissue culture medium osmolarity of 290 mOs/kg. We have measured a loss of water of dishes in our incubator of 75-100 μl/day, representing a 5% drop in volume evry day. Since only half the medium is changed at the weekly feedings, the medium continually becomes saltier. Presumably, the neurons are able to tolerate some slow variations in osmotic

pressure. We are currently attempting to answer the question: At what point does the medium become intolerable?

Fabrication

As noted in the last report, it was determined that an unacceptable amount of overhang was present around the periphery of the neuron wells being produced. The overhang is caused by undercut of the grillwork material in the final EDP etching step. Testing of chips with and without this overhang showed that outgrowth is rapid and abundant from non-overhung wells whereas the outgrowth from overhung wells is slow and sparse if present at all. As a result, a new process which produces wells guaranteed to have no overhang was created. Denoted the Zero Overhang via Removable Oxide (ZORO) process, the technique was proven and a run of dummy probes made with this process proposed.

Immediately after the submission of the last quarterly report, processing of the dummy ZORO probes began. An attempt was made to use a process which would eliminate the need for performing lithography on a membrane. Unfortunately the results of this run were far from adequate. Attempts to protect the already defined wells from the main cavity EDP etching failed and the lateral etching of the EDP, although slow, was more than fast enough to extend the walls neuron wells beyond the extent of their overlying grillwork. The result was a series of trapezoidal holes sitting on a large membrane. Not very useful.

Following this failure the decision was made to alter the fabrication process to permit steps that included performing lithography on the silicon membrane. While these steps are undesirable as they greatly increase the chance of breaking the membranes and even possibly shattering the wafer, the process as a whole is known to produces devices. In this case, hundreds of devices were successfully fabricated. Of a possible 518 dummy probes, 442 made it through all of the fabrication steps intact and with grillwork. Of these, more than 200 were deemed to be in near perfect to perfect condition. The other 200 or so had small imperfections such as one or two wells missing grillwork. Still, their condition was more than adequate for them to act as test instruments for the development of new implantation or mounting procedures. With the run completed, a batch of the dummy probes were separated from the wafer and sent to Rutgers for *in vivo* implantation.

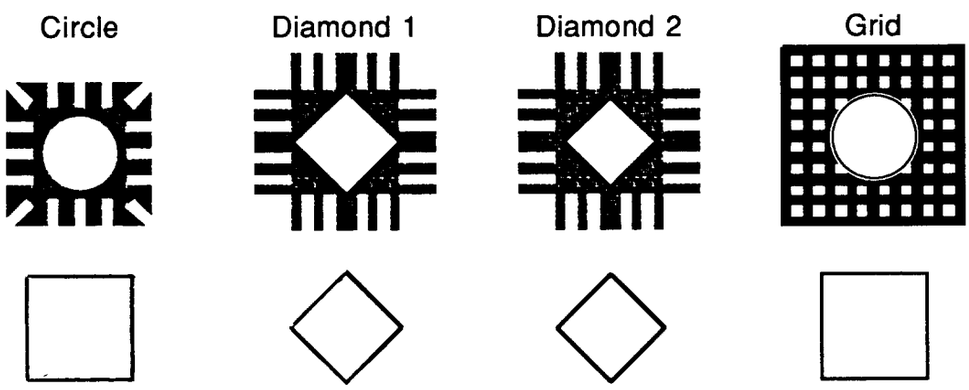
With the batch of dummy ZORO neuroprobes completed the next step was to create real ZORO neurochips that would hopefully allow the first neurophysiology to be done. Following a process very similar to that used to create the dummy ZORO probes, the chips were created without any major problems being encountered. Yield from the wafer was nearly 75% (approximately 30 chips), a new best for any of the neurochip processing runs. About 60% of these were near perfect to perfect dies. The remaining 40%, as in the case of the dummy probe run, has small imperfections such as one or two wells missing its grillwork. Once diced, the dies were prepared for neurophysiology. Unfortunately these chips were not destined to be the ones from which the first recordings would be made. During initial testing of the chips, two major problems were found. The first involved their electrical characteristics and the second the ability of their grillwork to prevent the escape of the implanted neurons.

The electrical problem is one of too much capacitance. It has been found that the capacitance of a bond pad is much greater than the coupling capacitance of the exposed portion of the electrode at the bottom of well. The result is a capacitive divider in which the majority of a signal is lost. At present we are attempting to salvage some chips by trying to remove the bond pads and connect directly to the electrode leads. So far we have had limited success. Platinization of the electrodes has also been found to help as it increases the coupling capacitance of the electrode at the bottom of the well, reducing the loss due to the capacitive divider.. In future versions of the chips/probes, this problem will need to be addressed by increasing the thickness of the insulation between the electrodes and the silicon substrate, by minimizing the size of the bond pads, and by optimizing the platinization of the well portion of the electrode.

The second problem, the ineffectiveness of the grillwork in keeping the neurons secured in the wells, is due to the "octopus" nature of the neurons. As the neurons grow, they extend processes out of the well through the holes in the grillwork. The processes move by gripping the substrate and pulling themselves along. This pulling action also draws the neuron away from the bottom of the well up towards the grillwork. Ideally the hole in the grillwork through which the process escaped will be much smaller than the neuron and it

will be unable to squeeze through and thus it will trapped within the well. Unfortunately, the neuron is basically a sack of water that can deform itself such that it can fit through very narrow openings much as an octopus can pull itself through an orifice the size of a keyhole. It has been guessed that holes smaller than 3μm in diameter are needed to prevent the neurons from escaping. Before now, this problem had not yet presented itself due to the lackluster rate of outgrowth that we have previously achieved. Also, SCG neurons do not escape as easily as hippocampal neurons.

To solve this problem, a run of dummy chips has been initiated that will produce an array of wells with several different types of grillwork. From these we hope to find a design capable of preventing the escape of neurons, and that can be reliably fabricated at the bottom the a 500μm cavity. As noted before, it has been suggested that 3μm lithography may be necessary to ensure that the neurons will not be able to squeeze their way out of their wells. However, such small feature will be extremely hard to reliably fabricate on a membrane at the bottom of a cavity. Before committing to such difficult constraints, the following designs will all be tested and the best one adopted as the grillwork of choice for future generations.



The first row of pictures shows the design which will be etched into the nitride to create the grillwork itself. The black indicates where the nitride will be left and the white where it will be etched away. As one can see, all nitride around the wells will be removed with the little that remains being that which forms the grillwork.

The second row of pictures indicates the shape of the hole that will be etched in the oxide layer through which the EDP will etch the underlying silicon substrate to form the neuron wells. The circle design is the most straightforward. The size of the opening in the oxide layer is designed to be the same as the desired size of the top of the well. Providing that alignment and exposure are well controlled this mask will produce wells that are exactly as designed. Unfortunately, the conditions for exposure at the bottom of a large cavity are far from ideal making it unlikely that such a mask will produce good, reliable results. For the purpose of our tests, however, it may provide us with a feel for the minimum size hole through which a neuron can escape.

The two diamond designs are very similar to the circle design with the major difference being the manner in which the oxide is patterned. For the diamond 1 design, the four points of the diamond pattern in the nitride extend out such that they are even with where the slots in the nitride begin. The oxide mask is then the same size and shape as this diamond. The idea behind this mask set is that over-exposure in the oxide definition step will have no effect in the final shape of the well. As long as the pattern in the photoresist is not grossly deformed, the oxide that is removed will be precisely that which is within the diamond pattern in the nitride. When etched by the EDP, the edges of the resulting well's opening will nominally be even with where the slots in the nitride begin. In this sense, these masks create a self-aligned well. Controlled over-etching can then slowly increase the size of the well until its edges extend sufficiently past the beginning of the slots. The drawback of this design is that it allows for little error in the nitride lithography step. If, for instance, exposure distortion of 1μm occurs (ex. a 10 μm line loses 1μm on each side resulting in an 8μm line. This much distortion is realistic for lithography at the bottom of a 500μm cavity) then the initial size of the well will be 2μm beyond the start of the nitride slots. This may be too much. Diamond 2 provides a fix for this possible problem. In this case the points of the diamond opening in the nitride are 2μm shy of where the slots in the nitride begin. Again, the oxide definition mask is the same size as this diamond. This allows for a substantial amount of distortion to occur before the initial well opening extends beyond the start of the slots. Thus, the final size of this well will always be determined by controlled EDP over-etching and not lithography.

Finally, the grid mask set incorporates the self-aligning and controlled etching techniques of the two diamond designs while physically limiting the maximum size of the holes in the grillwork. All four mask sets were created in four feature sizes, i.e. four slot and line sizes. The sizes range from 3μm to 10μm depending on the mask set. Dummy chips using these mask sets have been fabricated will soon be stuffed with neurons to determine which design is the most efficient neuronal jailer.

Imaging of fluorescent hippocampal neurons in slices

Phototoxicity

We would like to observe the growth of neurons from the probe wells, as they integrate with the cultured hippocampal slice. This requires a means of making the probe neurons stand out from the slice neurons. In previous updates, we have described our success with staining dissociated hippocampal neurons with DiI, in such a way that they survive in culture and remain stained for many days. As mentioned in the last update, we have tackled the problems of non-specific staining of slice neurons with great success.

Recently, we have addressed the problem of phototoxicity. Although we use 'vital' dyes that are chemically inert, when illuminated with a bright light source, all fluorescent dyes generate cytotoxic byproducts to some extent, thought to be free radicals or singlet oxygen. We have noticed that the amount of illumination that is required to photograph cultured neurons stained with DiI is enough to cause them to die within a day. This makes it difficult to observe and document the growth of probe neurons over a period of several days, without killing them.

To assess the extent of the problem we conducted an experiment in which cultures of stained neurons were exposed to the mercury arc lamp (using the standard rhodamine filter cube) for varying amounts of time. The cells were grown on numbered grids, to allow us to return to the illuminated region the next day and check survival.

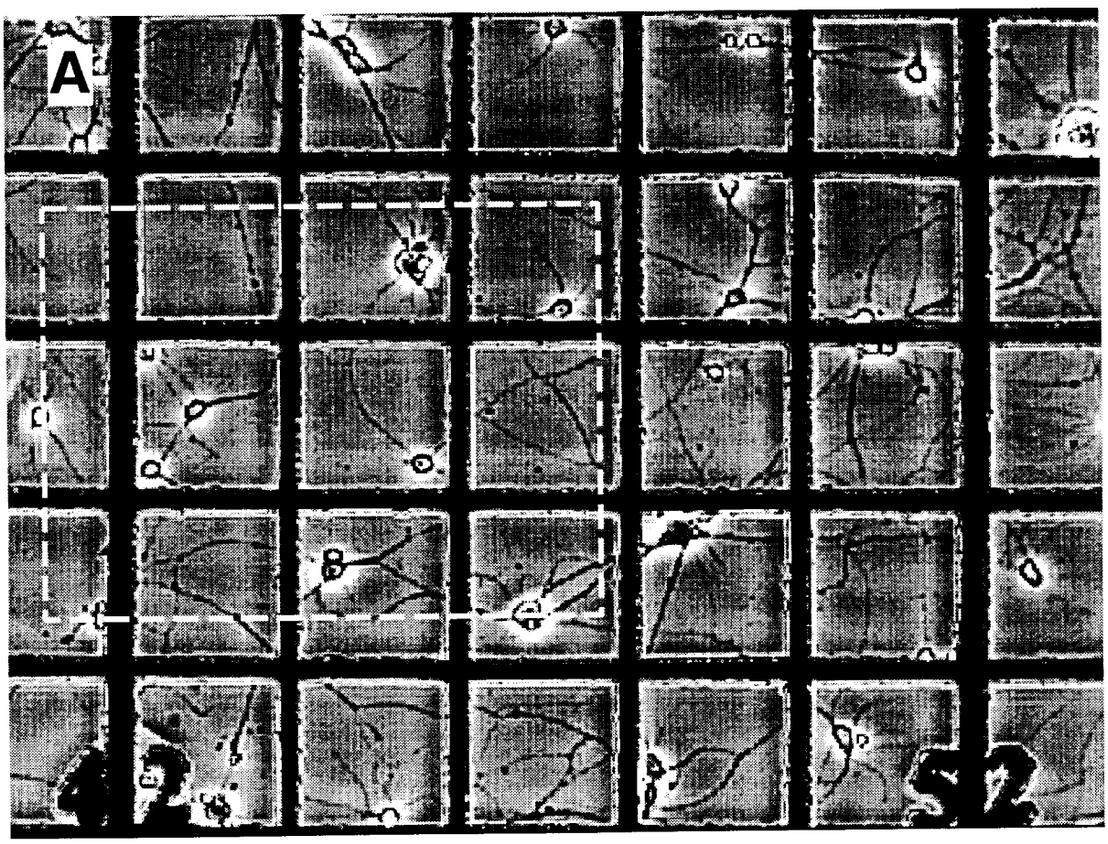
Hippocampal neurons from E17 rat embryos were dissociated and stained with either DiI (40 μg/mL, 10 min @ 37°C) or just vehicle (0.1% dimethylformamide, 0.0025% Pluronic F127), washed according to our previously described protocol, and plated on grid dishes coated with polylysine and laminin at approximately 300 cells/mm^2. On day 3 in culture, after the cells had established themselves and grown numerous processes, a 5x5 set of 100 μm squares was illuminated for times from 10 sec to 6 min. Duplicate cultures were used for each exposure. The exposed region, and a representative region on the same dish not exposed to the arc lamp, were photographed with a video

camera (using the tungsten lamp for phase-contrast imaging) just before and one day after exposure.

Exposed regions 'stained' just with vehicle (no dye) showed no phototoxicity, even with a 6-minute exposure. In contrast, dyed cells exposed to the arc lamp for as little as 10 seconds showed substantial deterioration of the cells' health, manifested by beaded processes. A 30-second exposure killed all the cells exposed, indicated by disintegration of processes, bright nuclei and round cell bodies. On the following pages are shown (A) the region of a culture to receive a 1-min exposure, just before exposure, (B) the same region one day later, (C) a fluorescence image of the subregion outlined in (A) captured during the exposure, and (D) a control region from the same dish, captured the day after exposure, showing that, except for the unfortunate exposed neurons, the culture is in good health. All but one of the exposures were made using a 20x, 0.75 NA objective. In one case, a 10x, 0.25 NA lens was used for a 10-sec exposure, resulting in no ill effect on the cells. The reduction in phototoxicity with a lower numerical aperture lens is not a benefit, since longer exposures are needed to make an equally bright image on film. It seems, brightness and phototoxicity are proportional, although we have not carefully quantified fluorescence brightness because we are limited by the sensitivity of our video camera.

There are a number of potential solutions to the problem of phototoxicity. We can use faster film and reduced exposure times, sacrificing some resolution. We could image outgrowth with a high-resolution integrating video camera, which the Pine lab is not currently set up to do. We could try new dyes, in the hope that they will be less phototoxic.

As a first step, we are working on imaging the stained neurons using a microscope designed to have greatly reduced phototoxicity by taking advantage of a 2-photon effect, described below. The microscope, part of the Biological Imaging Center at Caltech which is headed by Prof. Scott Fraser, is a Molecular Dynamics Sarastro 2000 confocal microscope that has been modified by Steve Potter to use a high-intensity pulsed infrared (IR) laser as a light source.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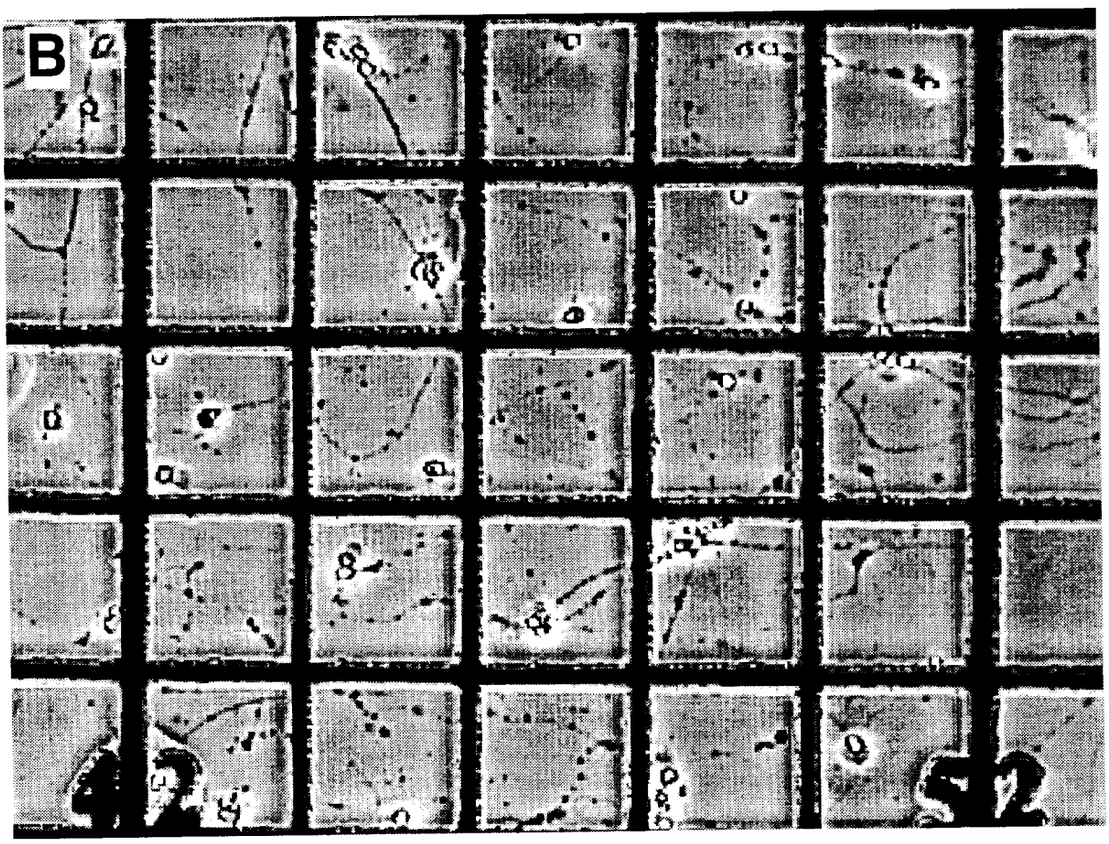
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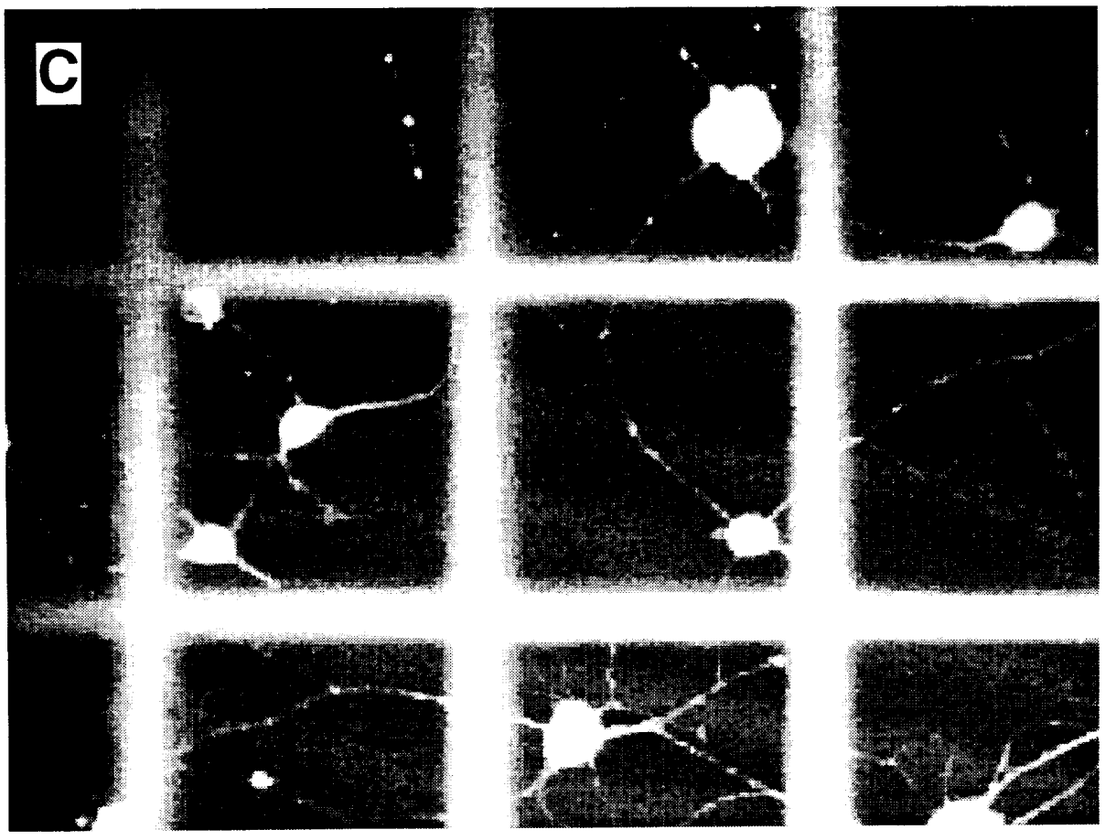
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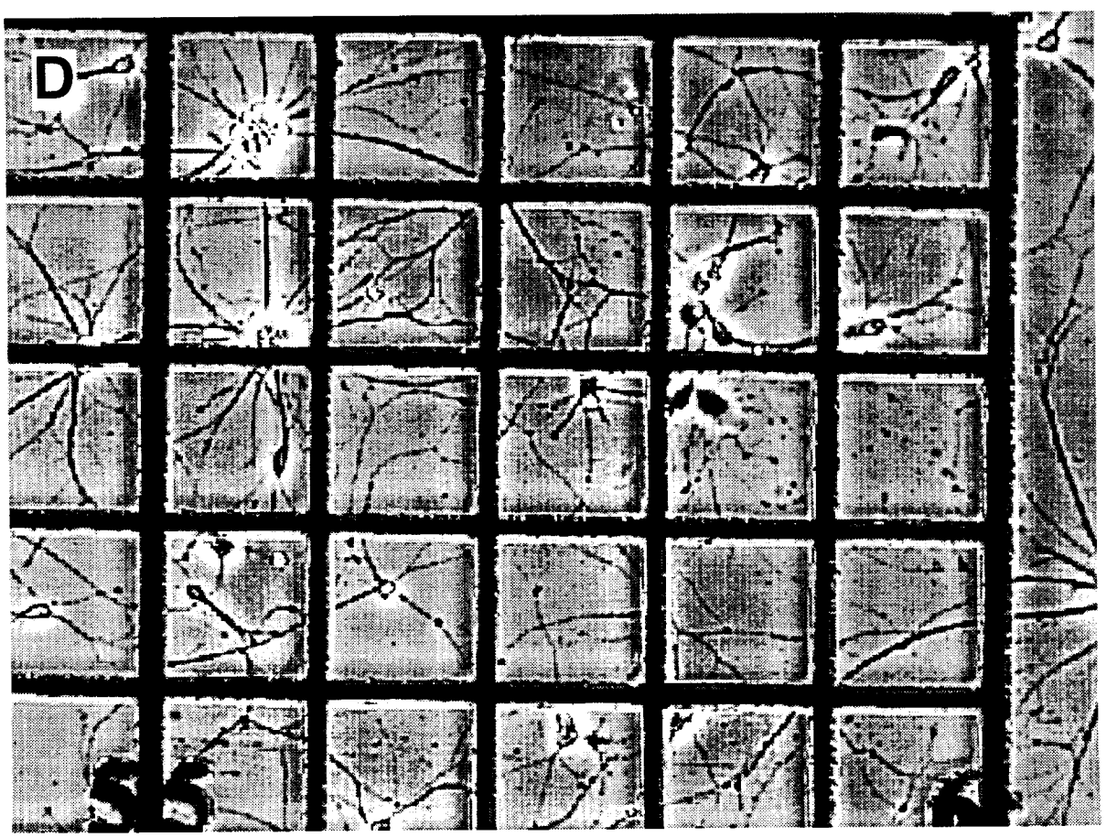
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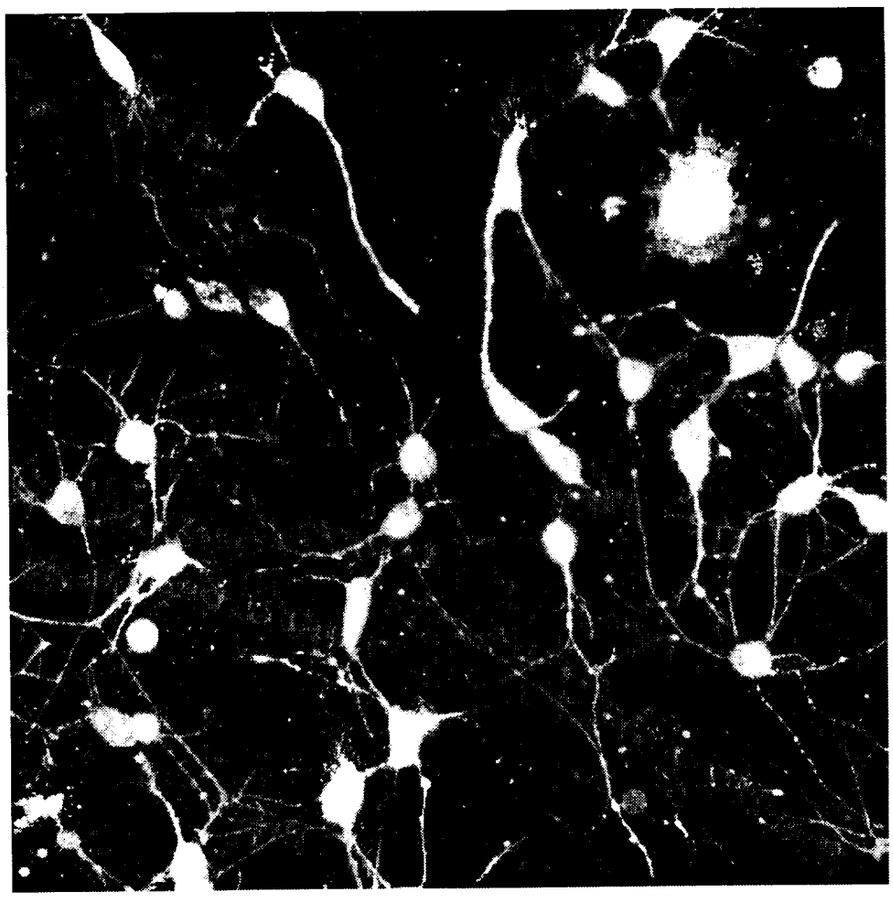
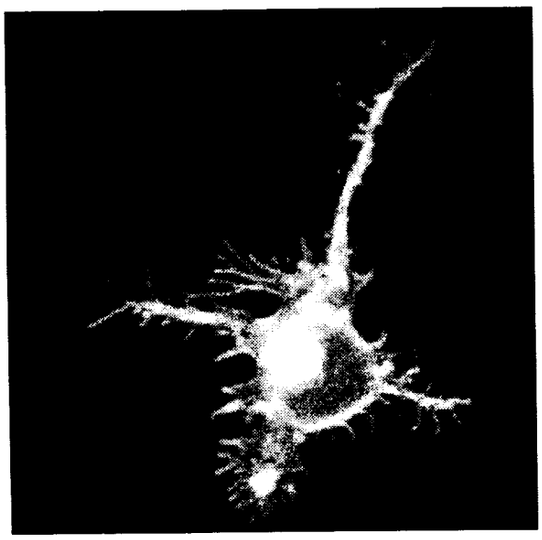
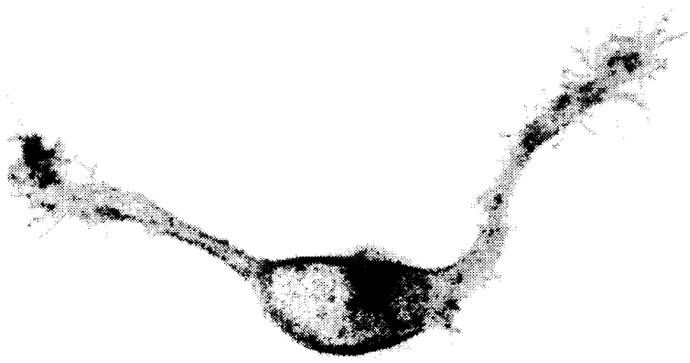
2-Photon fluorescence microscopy was recently pioneered by Watt Webb and his co-workers at Cornell (reviewed in Williams et al. 1994). By using a pulsed IR laser, fluorescence is generated only at the focus of the beam, where the intensity is great enough that two IR photons can hit a dye molecule at the same time (within 1 fsec), summing their energy to roughly that of one visible photon of half the wavelength. At all points further than the diffraction limit of the optics being used (typically 1 μm or less), the photon flux is too low for a 2-photon effect. Thus, the tissue suffers phototoxic damage and photobleaching only for the brief moment (typically 5 μsec) that the laser scans through any point. Above and below the plane of focus, there is no damage at all, since there is no fluorescence. Normal confocal microscopes use a pinhole to reject out-of-focus fluorescence, but no pinhole is needed for 2-photon microscopy, since the fluorescent light is being emitted by a diffraction-limited spot. Thus, all of the emitted fluorescence that can be collected by the objective is used to produce the final image. And this final image has a greatly improved resolution in the direction of the optical axis, compared to even confocal microscopy, due to the complete lack of out-of-focus fluorescence. Thus, serial optical sections of thick specimens show unprecedented detail.

Although the peak energy of the IR pulses is large (tens of kilowatts), because of the short duty cycle, the mean energy is only a few milliwatts, and this does not lead to any substantial heating of the specimen due to IR absorption.

We have recently finished the 2-photon modifications of the microscope and taken the first images of stained neurons, and the microscope seems to be performing as expected, although fluorescence of DiI stained neurons is not as bright as we would like it to be. On the following page are some sample images from our 2-photon microscope. The top picture shows living DiI-stained hippocampal neurons after three days in culture. Ten scans were averaged to reduce the noise in this image. We have tried other vital dies and have had brighter staining with DiO, DiA, and BODIPY ceramide, than with DiI. The bottom images are of living DiA-stained neurons, 6 hours after plating, with no signal averaging necessary. (The greyscale of the lower-right image has been inverted to show detail.)







Slice studies

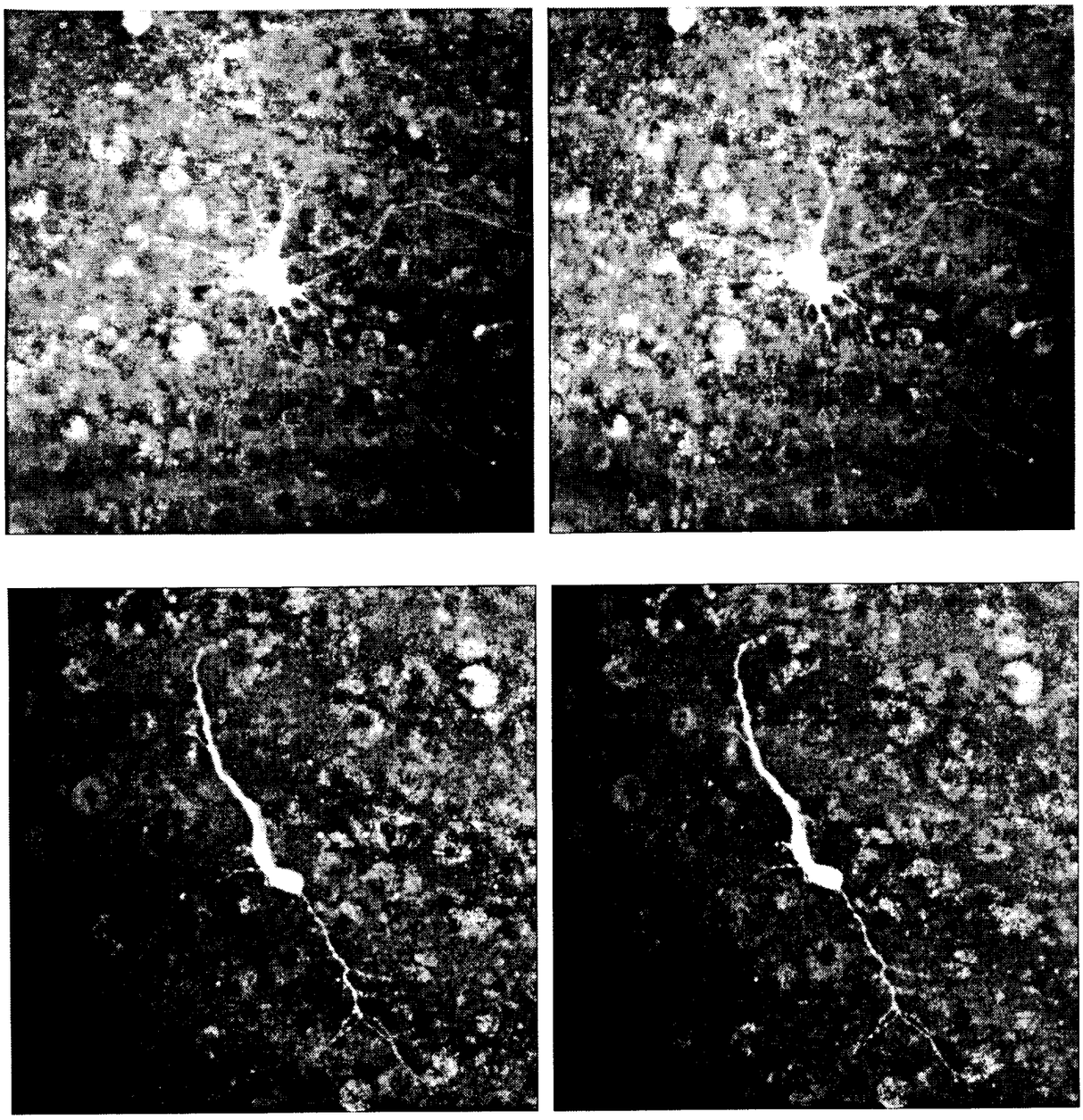
We have assessed the ability of dissociated embryonic hippocampal cells to integrate with our slice cultures by plating stained cells directly onto the surface of the slice. This allows testing of dye brightness, phototoxicity, and other variables related to staining and imaging, without the difficulty of preparing probes with stained neurons in their wells. When plated at a low density (approx. 20,000 cells/mL, 5 µL/slice) a few tens of cells will settle on the slice far enough from each other that their processes can easily be followed back to the originating cell body.

We have observed no dye transfer to adjacent neurons, although the stain from dead cells does get taken up by glial cells in the slice. The brightness of DiI-stained neurons growing on the slices fades about as fast as for neurons plated on petri dishes, with processes still being clearly visible after a week or more. There is substantial outgrowth of stained cells into the slice even after one day. The following figures are stereo images of neurons two days after plating onto slices, taken with the 2-photon microscope. They are produced from stacks of 30 3-micron sections, extending 50-100 µm into the slices. The hippocampal slices were prepared from postnatal day 7 rat pups, and grown a day in culture before the stained neurons were applied. The cells are already displaying stellate and pyramidal morphologies typical of hippocampal neurons, with fine processes hundreds of microns long.

We plan to conduct studies of phototoxicity soon, although preliminary observations showed no acute effect on living neurons after prolonged (several minutes) scanning. We are also working on improving the optical throughput of the system to improve brightness of sub-optimal dyes like DiI.

Reference:

Williams, R. M., D. W. Piston, W. W. Webb (1994). "2-photon molecular-excitation provides intrinsic 3-dimensional resolution for laser-based microscopy and microphotochemistry." *Faseb J.* 8: 804-813.



In Vivo Studies

Long Term Labeling

For examining the long-term survivability of hippocampal cells labeled with a marker in future probe experiments dissociatied fetal hippocampal neurons were stained with DiI and the cell suspension was grafted into 2 month-old rats. Cells stained with DiI were examined 6 months after transplantation. Several neurons and a few axons were visible within grafts.

Staining procedure:

1. DiI stock solution (40 mg/ml DiI/DMF + 2.5% pluronic F127) and 2ml serum-free Neurobasal/B27 were warmed in 37°C water bath.
2. 1 µl warmed DiI stock solution was pipetted into 2ml warmed media.
3. Suspension from six to eight hippocampi of 17 day rat embryos was pipetted into stain media.
4. The tube with suspension was placed into the incubator for 60 min, and swirled every 5-10 min.
5 The tube with suspension was centrifuged at 200g for 3 min.
6. Supernatant was pipetted off, 1 ml Neurobasal/B27 medium was added and the cell pellet was resuspended.
7. Steps 5 and 6 were repeated and cells were left at room temperature after additional centrifugation till transplantation.

Transplantation procedure:

Sprague-Dawley rats were anesthetized with a mixture of 4 mg/kg of ketamine (25 mg/kg), xylazine (1.3 mg/kg) and acetopromazine (0.25 mg/kg) and fixed in a stereotaxic frame. 0.3-0.5 mm^3 of suspension taken from pellet were transplanted into the left and right hippocampus by a microsyringe after opening the skull.

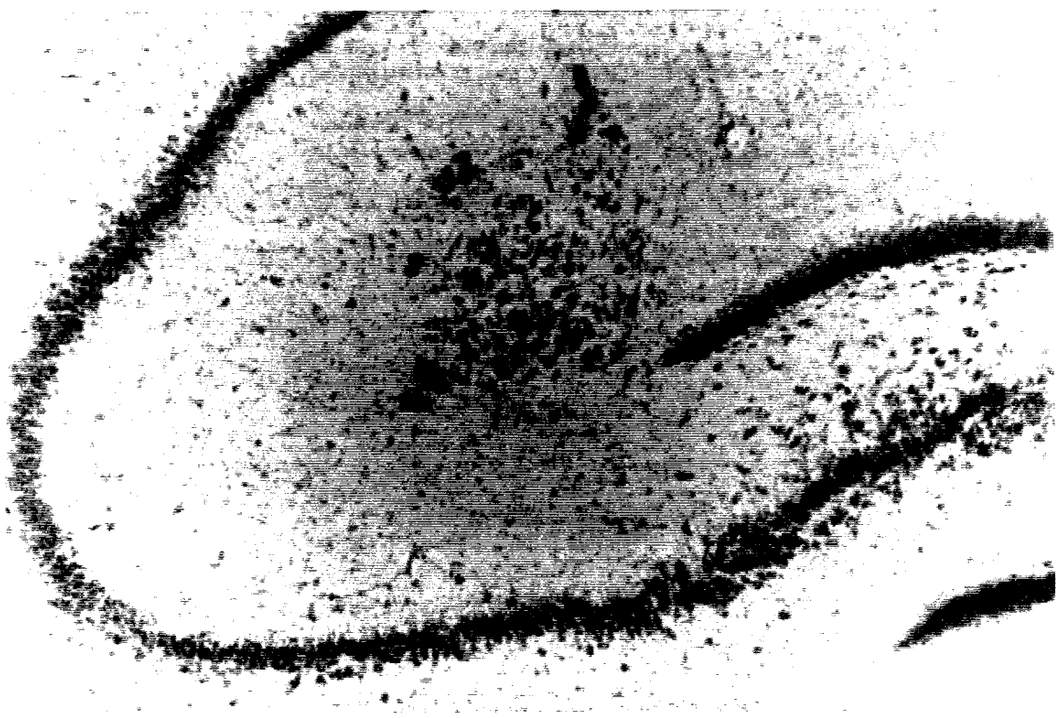
For verification of the transplants rats were deeply anesthetized and

perfused with 200 ml of saline followed by 10% of buffered formalin. Then brains were cut on a vibratome (60 μm sections) and examined under the fluorescent microscope.

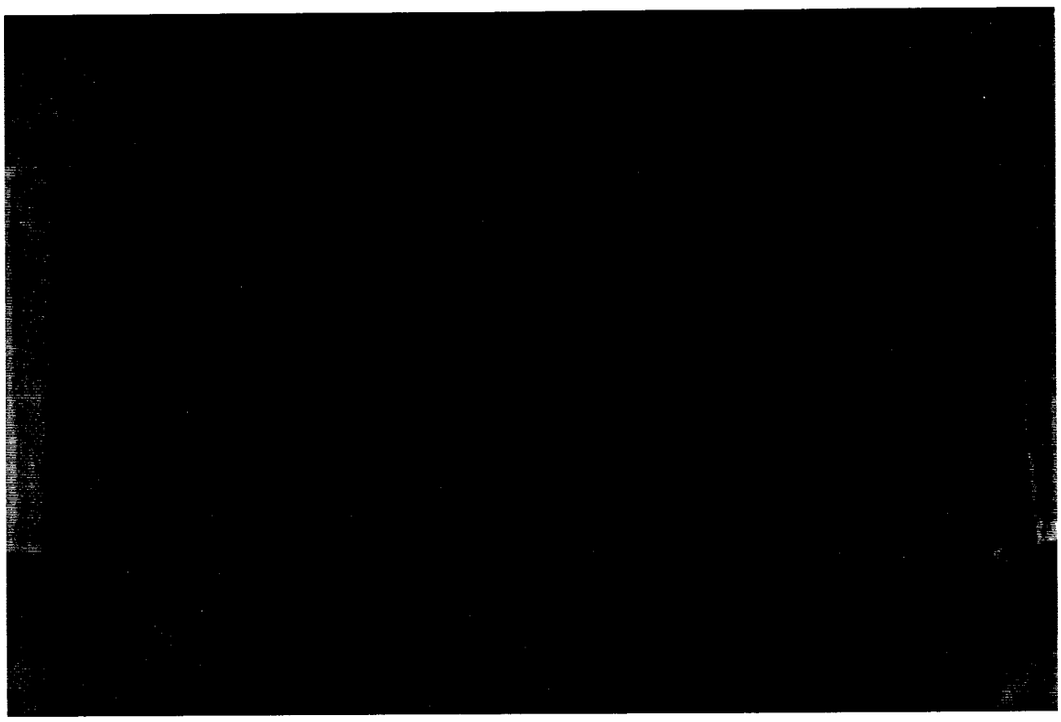
Results:

Eight rats received stained suspension cell transplants. Part of the suspension used for transplantation was also used for culturing neurons on glass surfaces for 1-3 days. About 70% of the cells in the tissue culture were stained on the first or second day of culturing.

After six months of survival transplants were found in all tested rats. As in previous cases they had a volume about 1mm^3. A Nissl stained section is shown below.



Similar to grafts 1month after survival, as reported in a previous progress report, neurons with DiI fluorescence were also seen after 6 months period of survival. The dye concentrated in the cell membrane, in line with the lipophilic nature of DiI, as shown in the fluorescence micrograph below.



Axon bundles crossing the graft-host interface were also noticed. However, individual axons were rarely seen and they could not betraced back to the parental neurons because of the strong backgound fluorescence. However, with confocal microscopy such retrograde tracing may work. Even at 6 months, some extracellular crystals were still present aswere several labeled glia. Occasionally, glia and neurons were also labeledoutside the graft. These observations suggest that some surviving cells may not have been labeled in vitro, but incorporated the dye from the DiI crystals which were inadvertantly injected along with the cell suspension.

Culturing with new dummy probes

To date, we have done two experiments with new dummy probes. In the first experiment fungal infection occurred likely due to the fact that the culture rig was not in use for 4 weeks. In the other experiment the neurons

were observed within wells for several days, but no growth of processes was observed. Several of the neurons escaped from the wells withing 12 hours. One possible reason for failing to see outgrowth is that the probes were covered only by polylysine. As we learned from our colleagues at Caltech, polylysine alone may not be a sufficient surface for hippocampal cells. In future experiments, we are going to use both polylysine and laminin to coat the probes before culturing.